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An investigation into the mechanism of TMIGD1-mediated signal transduction pathway in human epithelial cells

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AN INVESTIGATION INTO THE MECHANISM OF TMIGD1-MEDIATED SIGNAL TRANSDUCTION PATHWAY IN HUMAN EPITHELIAL CELLS

by

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ABSTRACT

Dysregulation of protein expression, in particular expression of proto-oncogenes and tumor-suppressor genes whose function play key roles in cell growth, adhesion and migration, are hallmarks of human malignancies.

Transmembrane and immunoglobulin-containing domain 1 (TMIGD1) was recently discovered as a cell adhesion molecule (CAM) that plays an important role in epithelial cell function by regulating epithelial cell polarity and adhesion. The extracellular domain of TMIGD1 contains two Ig domains that are involved in cell-cell interaction, followed by a transmembrane region and short cytoplasmic domain with potential to relay signal transduction. Our further investigation demonstrated TMIGD1 is downregulated in human colon cancer, suggesting a potentially important role for TMIGD1 in the regulation colorectal cancer. However, the molecular mechanisms of TMIGD1-mediated signal transduction, which could relay its function in epithelial cells, are not known.

Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, we have identified moesin as a possible TMIGD1 binding protein. Moesin, a member of the Ezrin/Radixin/Moesin (ERM) family of proteins, is upregulated in human tumors.

Moesin stimulates cell migration, tumor invasion, adherence and modulates cytoskeletal actin assembly. Similar to other ERM family proteins, moesin contains an N-terminal FERM domain, which binds to transmembrane proteins, and a C-terminal C-ERMAD domain, which binds F-actin. The overall goal of this study was to determine the binding of moesin with TMIGD1 and the specific domain involved in mediating the binding of moesin with TMIGD1.

Our study *in vitro* and *in vivo* binding assays demonstrate that moesin interacts with the cytoplasmic domain of TMIGD1 via its FERM domain. Moreover, we demonstrate TMIGD1 interaction with moesin inhibits phosphorylation of moesin, indicating that perhaps TMIGD1 inhibits tumor cell migration through inhibition of phosphorylation of moesin. Additionally, TMIGD1 alters cellular localization of moesin, suggesting that altered cellular localization by TMIGD1 could account for inhibition of phosphorylation of moesin. We propose that TMIGD1 sequesters moesin near the cell membrane, preventing its interaction with PIP2, which is required for its phosphorylation and hence inhibits moesin activation.

Altogether, the data presented in this work identifies moesin as a key signaling component of TMIGD1. Moesin directly interacts with TMIGD1 via its FERM domain. Recruitment of moesin to TMIGD1 blocks phosphorylation of moesin, suggesting that TMIGD1 exerts its effect in tumor cells in part by inhibition of moesin activation.

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LIST OF ABBREVIATIONS

amp..... Ampicillin

BSA..... Bovine Serum Albumin

CAM ..... Cell Adhesion Molecule

Cyt-TMIGD1 ..... Cytoplasmic TMIGD1

DMEM ..... Dulbecco's Modified Eagle Media

DTT..... Dithiothreeitol

E-cadherin ..... Epithelial Cadherin

ECL..... Enhanced Chemiluminescence

ECM..... Extracellular Matrix

ERM..... Ezrin / Radixin / Moesin

EV ..... Empty Vector

FA ..... Focal Adhesion

FAK..... Focal Adhesion Kinase

FBS .....Fetal Bovine Serum

GFP .....Green Fluorescent Protein

GST..... Glutathione S-Transferase

HEK-293T..... Human Embryonic Kidney

HRP..... Horseradish Peroxide

Ig .....Immunoglobulin

IGPR-1 .....Immunoglobulin-Containing Proline Receptor 1

IgSF..... Immunoglobulin Super Family

IP ..... Immunoprecipitation

IPTG ..... Isopropyl β-D-1-thiogalactopyranoside

LB ..... Lysogeny Broth

LC-MS/MS ..... Liquid Chromatography-Tandem Mass Spectrometry

MMP ..... Matrix Metalloproteinase

P/S ..... Penicillin / Streptomycin Solution

PBS ..... Phosphate Buffered Solution

PEI ..... Polyethlyenimine

pH ..... Potential of Hydrogen

PIC ..... Protease Inhibitor Cocktail

PVDF ..... Polyvinylidene fluoride

RKO ..... Rectal Carcinoma

RPMI ..... Roswell Park Memorial Institute Medium

SDS ..... Sodium Dodecyl Sulfate

SDS-PAGE ..... Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

TEER ..... Trans-Epithelial Electrical Resistance

Thr ..... Threonine

TMIGD1 ..... Transmembrane and Immunoglobulin Domain-containing 1

WCL ..... Whole Cell Lysate

INTRODUCTION

Tumorigenesis

The earliest stages of tumorigenesis occur following deviance in otherwise healthy cells. Deviance often begins with mutations in genes that govern key aspects of cell proliferation, apoptosis, migration, differentiation and cell adhesion. Accumulation of these genetic mutations lead to paramount changes in cells, causing what have been described as the six benchmarks of human malignancies: i) growth signal self-sufficiency, ii) anti-growth signal insensitivity, iii) evasion of apoptosis, iv) limitless replicative potential, v) sustained angiogenesis and vi) tissue invasion and metastasis (Hanahan & Weinberg, 2000). Study of tumor-suppressor genes and proto-oncogenes promptly reveals their roles in attaining these benchmarks.

As the name indicates, tumor-suppressor genes are integral to preventing tumor development in normal cells. There are many tumor-suppressor genes in the human genome operating by various mechanisms of function. The function of these ubiquitous genes follows four main tenets: i) suppression of cell division, ii) induction of apoptosis, iii) stimulation of DNA repair and iv) inhibition of metastasis (Sun, 2010).

Suppression of cell division is accomplished by several well-known tumor-suppressor genes. The tumor-suppressor Rb binds to transcription factors to directly inhibit cell division (Müller & Helin, 2000). p53 plays numerous roles in tumor-suppression. In apoptosis, p53 induction mediates release of cytochrome-C from the mitochondria to initiate an apoptotic cascade while in DNA repair, p53 interacts with the tumor-suppressor BRCA to facilitate nucleotide excision repair (Benchimol, 2001;

Hartman & Ford, 2003). A final example, NME1, facilitates increased binding to the extracellular matrix (ECM) to impede tumor migration and metastasis (Novak et al., 2015). These serve as only a few classic examples, as there are numerous tumor-suppressors employing a multitude of mechanisms.

Proto-oncogenes in their normal state generally are responsible for regulation of mitosis and cell growth, however, following specific mutations, proto-oncogenes become oncogenes and the processes previously checked become dysregulated. Processes left vulnerable following oncogene inception can be grouped into the following general categories: transcription factors, growth factors and their receptors, chromatin remodelers, cellular signal transducers and the regulation of apoptosis (Croce, 2008).

Cell Adhesion Molecules and Cancer Cells

Primary tumors can lead to dysfunction in their originating tissues, however, leading cause of cancer-related death is metastasis (Hanahan & Weinberg, 2000). Metastasis involves invasion and migration of cells, often driven by morphological change. CAM's are a chief mediator of the changes causing metastasis and include molecules in the families of cadherins, integrins, selectins and the immunoglobulin super-family (IgSF).

Integrins are dimeric transmembrane protein receptors that bind to the ECM. The heterodimers, which consist of an alpha and beta dimer, have at least 24 combinations composed from 18 alpha and 8 beta dimers (Madamanchi, Zijlstra, & Zutter, 2014). With this diversity, integrins elicit numerous intracellular cascades. These cascades are initiated by the extracellular integrin receptor binding to an extracellular protein ligand,

usually fibronectin, collagen or laminin, causing clustering of integrins. Signal cascades elicited by clustering often lead to recruitment of other membranous receptors or modulation of cytoskeletal components and creation of focal adhesions (FA) (Juliano, 2002). Although integrins lack kinase activity, recruitment of kinases such as focal adhesion kinase (FAK) allow for downstream phosphorylation of target proteins involved in cell migration and adhesion (Hermann et al., 2016).

The diverse signaling of integrins mediates cell survival, proliferation, migration and invasion, making integrins useful targets for oncogene manipulation in tumor cells. In normal cells, the binding of integrins to extracellular ligands promotes survival while lack of ligand binding elicits apoptotic stimuli (Desgrosellier & Cheresh, 2010). Although the diversely-functioning integrin receptors are not uniformly up or down-regulated in tumor cells, trends are observed. Because integrins are not oncogenes, and thus not inherently mutated in tumorigenesis, their regulation must altered by oncogenes. Oncogene regulation leads to upregulation in pro-survival, proliferative, migratory and invasive stimuli and down-regulation of ECM binding, specifically binding to laminin of basement membranes (Madamanchi et al., 2014).

Cadherins are a family of transmembrane glycoproteins that mediate calcium-dependent cell to cell adhesion. Of particular interest in tumors is epithelial-cadherin (E-cadherin). E-cadherin plays a critical role in cell-cell adhesion interactions. Extracellular domains of the protein form homophilic interactions with the extracellular domains of E-cadherins on adjacent cells (Roy & Berx, 2008). This extracellular binding leads to activation of growth inhibition cascades mediated by RTK and Src family kinases (N.-G.

Kim, Koh, Chen, & Gumbiner, 2011). This mechanism of inhibition is responsible for the phenomenon of contact-dependent inhibition. Mechanisms of contact-dependent inhibition are often diminished or totally impaired in tumor cells, leading to overgrowth.

Catenins are intracellular protein domains that interact with cadherins to mediate many signaling pathways. Catenins play an important role mediating the interaction of cadherins and the cytoskeleton of the cell, in particular the binding of actin. Catenins are sequestered inside adherens junctions in epithelial cells and mutations in tumor cells commonly lead to the loss of catenin sequestering (Farahani et al., 2014). Wnt signaling is a well-studied pathway which leads to the inhibition of beta-catenin degradation by APC complexes normally tagging cytoplasmic beta-catenin for degradation (Cavallaro & Christofori, 2001). Free cytoplasmic beta-catenin goes to the nucleus where it stimulates transcription.

IgSF CAM's are mostly type-1 transmembrane proteins with tremendous variability in their domains. IgSF proteins play a range of roles in cell-cell recognition as well as recognition of non-cellular matter in the ECM (Barclay, 2003). IgSF proteins may form homodimers with their counterparts on neighboring cells via their extracellular amino-terminus or heterodimers with different proteins, such as integrins, to mediate adhesion. The effects of the extracellular interactions are mediated by the intracellular C-terminus domain, facilitating changes in adhesion and morphology, among other functions (Okegawa, Pong, Li, & Hsieh, 2004).

Selectins are cell adhesion molecules playing a significant role in vasculature. They are transmembrane proteins with calcium dependent function. Selectins are

expressed in leukocytes and endothelial cells and play a significant role in the adhesion of leukocytes to the endothelial wall as part of leukocyte migration to sites of inflammation (Ley & Kansas, 2004). Selectins have three classes, P-selectin, L-selectin and E-selectin, all playing a role in hematogenous metastasis. Downregulation of selectins has been shown to correlate with decreased metastasis (Bendas & Borsig, 2012).

Ezrin / Radixin / Moesin Family Proteins

Ezrin, radixin and moesin (ERM) are part of the ERM family of proteins. These intracellular proteins are comprised of three parts: the N-terminus FERM domain, middle linker region and a C-terminus C-ERMAD domain. The FERM domain of all three homogeneous proteins is approximately 300 amino acids, consisting of three lobes, F1, F2 and F3. The central linker region is approximately 200 amino acids while the C-ERMAD is the shortest, at approximately 70 amino acids (Ben-Aissa et al., 2012).

ERM family proteins play a role in numerous cell functions such as adhesion, migration and invasion in tumor cells, secretion of matrix metalloproteinase (MMP), cell polarity, exocytosis and membrane rigidity (Li, Zhou, & Gao, 2015; Tsukita, Yonemura, & Tsukita, 1997; Vitorino et al., 2015). Of particular interest to this work is the protein moesin, which has been specifically linked to metastasis.

The diversity of ERM function can be well-appreciated through understanding the function of its domains. Ligands of the FERM domain are most commonly transmembrane proteins such as CD43, CD44, ICAM proteins and others. In binding to these ligands, ERM proteins are in indirect contact with the extracellular environment

and mediate cellular changes as dictated by the cellular environment (Pokharel et al., 2016).

The coiled middle segment of the protein links the FERM domain to the C-ERMAD domain. The C-ERMAD domain is responsible for binding to F-actin in the cytoskeleton of the cell (Figure 1). Through cytoskeletal F-actin binding, the C-ERMAD domain acts as the effector to the stimuli received at the N-terminus FERM domain.

Figure 1. Structure of moesin. Moesin is a 577 amino acid protein with three domains. At the N-terminus is a FERM domain spanning residues 1-316 and comprised of three lobes, F1, F2 and F3. The F3 lobe contains the lysine-rich PATCH region, which blocks FLAP. The inhibitory FLAP region, which blocks PIP2 access to POCKET, lies N-terminal to the C-ERMAD domain. Between lobes F1 and F3 is the POCKET region, which blocks the Thr558 from being phosphorylated. The FERM domain binds transmembrane proteins in the cell membrane. The middle linker region is composed of primarily alpha-helices. The C-ERMAD domain contains the Thr558 residue which is phosphorylated to confer activity. When activated, the C-ERMAD domain binds cytoskeletal F-actin (Figure adapted from Sauvanet, Wayt, Pelaseyed, & Bretscher, 2015).

In its basal state, the FERM domain of ERM proteins is bound to the C-ERMAD domain, resulting in autoinhibition of the protein and confinement to the cytoplasm. In moesin, activation occurs via phosphorylation of a threonine (Thr) located at residue 558 in the C-ERMAD domain (Figure 2). In the autoinhibited state however, Thr558 is blocked by a portion of the FERM domain and cannot be phosphorylated. To expose Thr558, positively charged lysine residues in the F3 lobe of the FERM domain, called PATCH, transiently bind PIP2. PIP2 binding to PATCH leads to displacement of a polar, acidic region called FLAP. FLAP is N-terminal of the C-ERMAD domain and blocks a second positively-charged PIP2 binding site called POCKET. POCKET is located in a cleft of the FERM domain between lobes F1 and F3. Following the transient binding of PIP2 to PATCH, POCKET stably binds PIP2 and is displaced, exposing Thr558 for phosphorylation (Ben-Aissa et al., 2012; Sauvanet et al., 2015).

Once activated, moesin migrates to the cell membrane where the FERM domain can interact with ligands and mediate its function in the cell. Although the function of moesin is diverse, there seems to be a specific correlation between the presence of moesin in tumor cells and degree of malignancy. Moesin has been shown to be upregulated in many cancers and suppression of moesin leads to decrease in several tumorigenic properties (C. Y. Kim et al., 2012). It has been demonstrated that moesin silencing results in less tumor migration and invasion, increased cellular adhesion and upregulation of E-cadherin (Li et al., 2015).

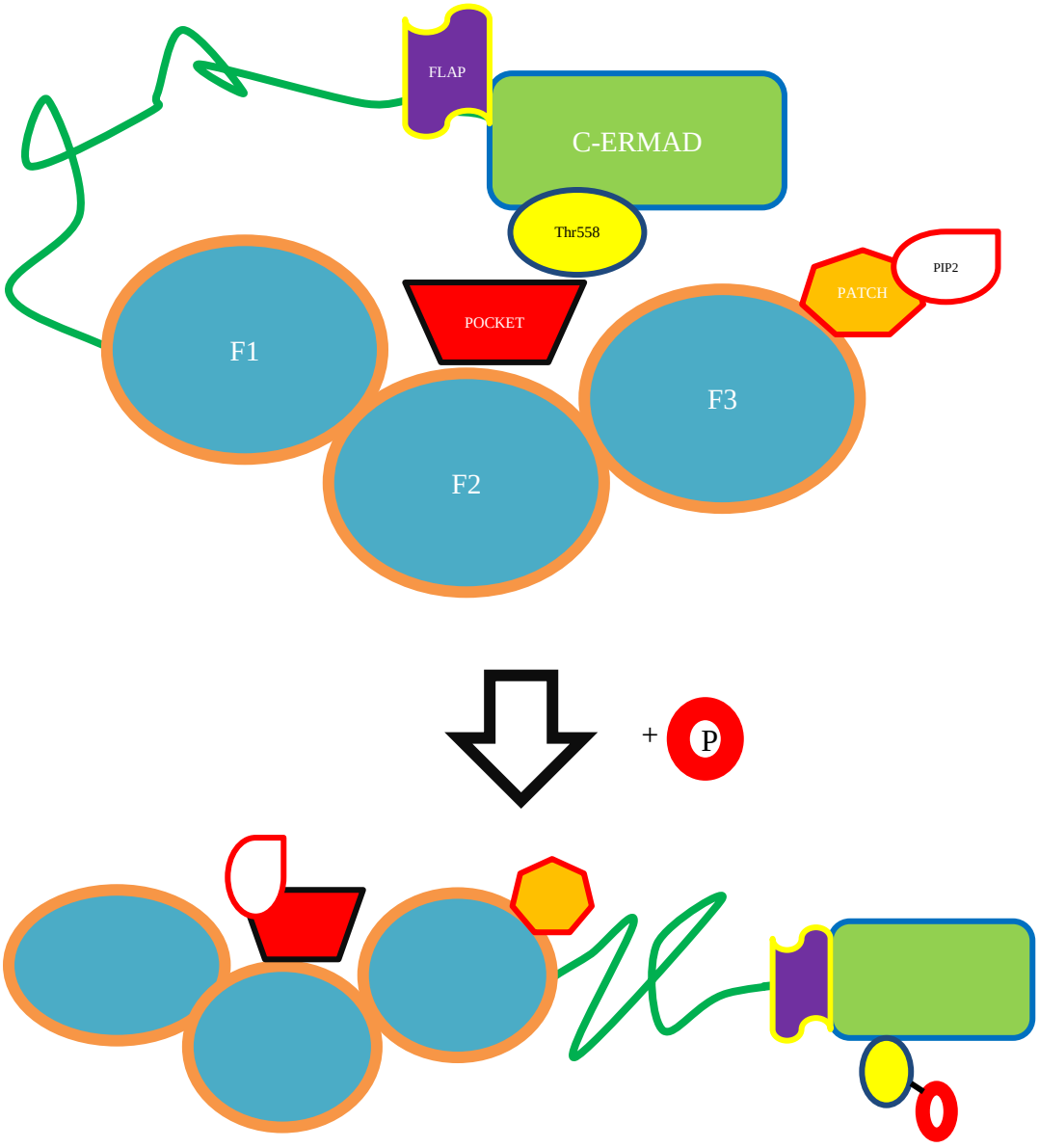

Figure 2. Activation of moesin. In its inactive, autoinhibited state, the C-ERMAD domain and linker region block most of the FERM domain. To expose the Thr558 residue for phosphorylation, PIP2 must transiently bind PATCH on the F3 lobe of FERM. FLAP, located N-terminal of the C-ERMAD domain, blocks POCKET, which is located in a cleft between the F1 and F3 lobes of FERM. FLAP dissociates from POCKET during transient PIP2 binding to PATCH, exposing POCKET for PIP2 binding. Stable PIP2 binding to POCKET exposes Thr558 on the C-ERMAD domain, allowing for phosphorylation, activation and opening of moesin.

Modulation of cell polarity is one of the means by which moesin elicits effects in tumor cells. By controlling the arrangement of cortical F-actin in the cell, moesin mediates invasiveness and mobility of cells (Abiatari et al., 2010). Thick cortical actin arrangements lead to cells with round morphology and disruption of FA's. The dynamic nature of moesin's regulation of cellular polarity was illustrated through real time imaging. Live confocal microscopy revealed uniform cortical moesin arrangement initially, followed by redistribution, yielding a moesin-rich, dome-like concentration of F-actin at the non-invading pole of the cell. Redistribution occurred after initial adherence and allowed blebs to form on the invading pole in the absence of actin filaments (Estecha et al., 2009).

Cell motility can also be controlled through direct moesin interaction with integrins. Under MAP4K4 control, moesin displaces the ERM-like protein talin from integrins. Talin displacement results in disruption of FA's and down-regulation of cell adhesion to the ECM (Vitorino et al., 2015).

Moesin has various distribution patterns throughout the cell depending on the state of the cell and whether or not it is tumorigenic. Investigation of cellular moesin distribution in oral squamous cell carcinoma revealed a spectrum of patterns ranging from membranous to diffusely-cytoplasmic. Cytoplasmic distribution correlated with increased malignancy (Kobayashi et al., 2004). All of this information taken together reinforces the diversity of moesin within the cell as well as the dynamic nature by which it mediates function.

TMIGD1 Family Proteins

TMIGD1 family proteins and immunoglobulin and proline rich receptor-1 (IGPR-1) were recently discovered as new class of cell adhesion molecules. IGPR-1 is highly expressed in endothelial cells and is required for endothelial barrier function and angiogenesis (Rahimi, Rezazadeh, Mahoney, Hartsough, & Meyer, 2012). TMIGD1 is a membranous protein with 262 amino acids and has two extracellular immunoglobulin domains, thought to dimerize to mediate function of the protein (Figure 3) (Arafa et al., 2015).

TMIGD1 functions as a CAM, leading to increased cell adhesion. Cytoskeletal rearrangement is one way in which TMIGD1 mediates its function. Expression of TMIGD1 results in a cortical distribution of F-actin in the cell, rather than a diffuse cytoplasmic arrangement when not expressed. This cytoskeletal arrangement may be responsible for the greater area covered by cells expressing TMIGD1 as opposed to a more compact presentation in cells expressing only an empty vector (EV). Expression of TMIGD1 has been shown to mitigate cell proliferation while overexpression leads to impaired migration in tumor cells. Lastly, it has been demonstrated that TMIGD1 plays a protective role in cells undergoing stress and increases survival when expressed. The caveat to this however is that under some circumstances, the injurious stimuli leads to ubiquitination of TMIGD1 and its subsequent breakdown, thereby impeding the protective mechanisms (Arafa et al., 2015).

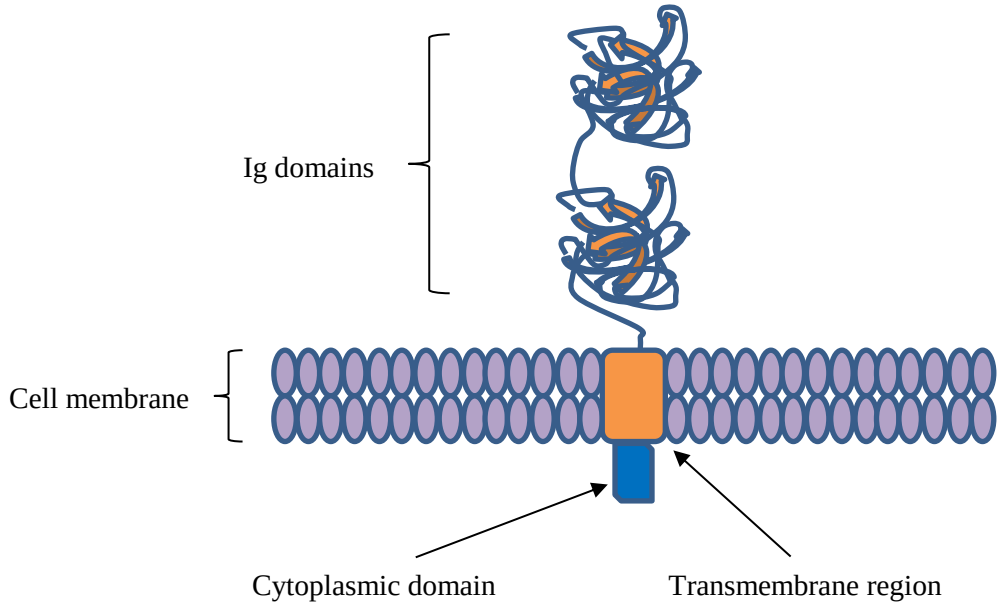

Figure 3. Schematic of TMIGD1. The novel adhesion molecule TMIGD1 possesses two extracellular Ig domains, a transmembrane segment and a short cytoplasmic domain. Unlike IGPR-1, the cytoplasmic domain of TMIGD1 is not proline-rich.

The overall goal of this project was to investigate the signaling mechanisms by which TMIGD1 elicits its effects in epithelial cells. Accordingly, a glutathione S-transferase (GST) pull-down assay consisting of the cytoplasmic domain of TMIGD1 (cyt-TMIGD1) fused to GST was used to identify signaling proteins that bind to TMIGD1 followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Moesin was one of the proteins identified as a putative TMIGD1 binding protein. Therefore, the primary objective of this thesis was to investigate the binding mechanism of moesin with TMIGD1 and its role in TMIGD1 signaling.

SPECIFIC AIMS AND OBJECTIVES

The following specific aims were designed to investigate the role of moesin in TMIGD1-mediated signal transduction:

Specific Aim I: Determine the *in vivo* and *in vitro* binding of moesin with TMIGD1.

Specific Aim II. Examine the hypothesis that moesin is recruited to TMIGD1 via its FERM domain.

Specific aim III: Determine whether binding of moesin to TMIGD1 regulates its phosphorylation and cellular localization.

METHODS

Cell Culture

HEK-293T cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 100 units/ml of penicillin and streptomycin solution (P/S) and 10% fetal bovine serum (FBS). Similarly, Roswell Park Memorial Institute (RPMI) media containing 100units/ml of P/S and 10% FBS was used to grow and maintain RKO cells. Unless otherwise noted, cells were maintained in 100mm adherent plates. Both HEK-293T and RKO cells were incubated in a humidified incubator at 5% CO_2 and 37°C. HEK-293T and RKO cells alike were transduced to overexpress TMIGD1 or empty vector pMSCV plasmids (herein referred to as EV) (purchased from Invitrogene) (Xueqing Zou et al., unpublished data).

Antibodies and Reagents

The rabbit polyclonal anti-TMIGD1(N8599) antibody was developed against the protein TMIGD1 (Arafa et al., 2015). Mouse monoclonal anti-moesin antibody (38/87) (cat# sc-58806) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti-C-MYC tag (9B11) antibody (cat# 2276) was purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-GFP tag (B-2) antibody (cat# sc-9996) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal anti-phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) antibody (cat# 3141) was purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal IgG anti-PLC γ1 antibody was purchased from Santa Cruz

Biotechnology Inc. (Santa Cruz, CA). All aforementioned antibodies were used as primary antibodies at a concentration of 2µL antibody/10mL of Block [2% bovine serum albumin (BSA) and 0.05% Tween-20 in Western Rinse] except anti-moesin (38/87). Moesin (38/87) antibody was used at a concentration of 40µL antibody / 10mL Block.

Two secondary antibodies were used in experimental procedures, both at the concentration 1µL antibody/10 mL Blotto [2% non-fat dry milk and 0.05% Tween-20 in Western Rinse]. Goat anti-mouse IgG horse radish peroxide-linked (HRP) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Goat anti-rabbit IgG HRP was made in-house.

Transfection

HEK-293T cells were transfected with GFP-tagged moesin and GFP-tagged EV controls using m-Emerald moesin N-14 and m-Emerald EV N-1 DNA plasmids, respectively. Plasmids were purchased from Addgene (Cambridge, MA). HEK-293T cells were grown to 90% confluence in 100mm plates and DMEM was aspirated. 4mL of FBS-free DMEM with P/S was then added to plates. 9µg of DNA and 30µL polyethylenimine solution (PEI) were added to 1mL of FBS-free DMEM with P/S in a 2mL Eppendorf tube, gently vortexed then equilibrated 15 minutes at room temperature. Following equilibration, the contents of one Eppendorf tube were added to each plate dropwise. Plates were incubated 6 hours, at which point 5mL DMEM with FBS and P/S was added each plate. 24 hours from the start of transfection, media was aspirated from plates and 10mL of new DMEM with FBS and P/S was added.

Western Blot Analysis

Cells were grown to 80-90% confluence in 100mm plates and placed on ice at benchtop. Plates were then rinsed with H/S buffer [20 mM Hepes (pH 7.4) and 150 mM NaCl]. After the second rinse, residual H/S buffer was aspirated and cells were lysed using EB lysis buffer [10mM Tris-HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100] with 4 mM Na3VO4 and 1.5% Protease Inhibitor Cocktail (PIC) [500 μM AEBSF, hydrochloride, 150 nM aprotinin, bovine lung, crystalline, 1 μM E-64 protease inhibitor, 0.5 mM EDTA, disodium, and 1 μM leupeptin, hemisulfate]. Whole cell lysates (WCL) were centrifuged and supernatants were transferred to new Eppendorf tubes. 5X sample buffer [bromophenol blue (0.25%)/dithiothreeitol (DTT) 0.5M/glycerol 50%, sodium dodecyl sulfate (SDS) 10%/Tris–Cl (0.25M, pH 6.8)] was added to WCL and tubes were incubated at 95°C for five minutes.

WCL's were resolved using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a methanol-activated polyvinylidine difluoride (PVDF) membrane. Membranes were blocked using Blotto then washed three times, five minutes each, in Western rinse. Membranes were subsequently incubated one hour in primary antibody solution, washed three times in Western rinse and incubated 45 minutes in secondary antibody solution. After secondary antibody, membranes were washed a final time and developed using enhanced chemiluminescence (ECL) (cat# 32106; ThermoFisher Scientific, Cambridge, MA).

GST-Fusion Protein Preparation

Fusion proteins were created in-house for use in GST pull-down assays. Three pGEX 4T vectors containing 1) GST control, 2) moesin amino acids 1-322 and 3) moesin amino acids 307-577 were used to create fusion proteins. E. coli bacteria containing these vectors were added to 15mL lysogeny broth (LB) medium containing 70µg/mL ampicillin (LB+amp) in a 15mL centrifuge tube and incubated in an automated shaker for 16 hours at 37°C. 3mL of LB medium was then transferred to a 500ml Erlenmeyer flask containing 100mL fresh LB+amp and incubated 4 hours in the shaker at 37°C. 400µL Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the flask and the solution was incubated another 4 hours. The contents of each flask were subsequently centrifuged and supernatant was discarded. Pellets were re-suspended in 5mL chilled phosphate-buffered solution (PBS) containing 50µL PIC. 1% Triton-X-100 in PBS was added to the re-suspended pellets and they were incubated on ice 30 minutes. Finally, solutions were centrifuged again and 500µL of 57% glutathione Sepharose 4B slurry beads purchased from GE Healthcare Life Sciences (cat# 17075601) were added to supernatant. After 4 hours incubation at 4°C, solution was centrifuged and supernatant discarded. Remaining beads were washed three times with chilled PBS+10µL PIC and stored at 4°C for GST pull-down assay. Fusion proteins created using pGEX 4T containing cyt-TMIGD1 were produced using identical protocol and provided courtesy of Xueqing Zou. Presence of desired fusion proteins was tested using SDS-PAGE and gel staining with Coomassie blue.

GST Pull-down Assay

Cells grown on 100mm plates were maintained until 90% confluent then lysed using EB buffer. WCL's were centrifuged and supernatants transferred to separate Eppendorf tubes. GST fusion protein beads were added to Eppendorf tubes in the following volumes, as indicated by test gel results: GST control: 25µL; GST-moesin (1-322): 120µL; GST-moesin (307-577): 60µL; GST-cyt-TMIGD1: 50µL. Eppendorf tubes were then incubated at 4°C for 4 hours. Beads were centrifuged and supernatant discarded prior to washing beads three times with 500µL EB buffer + 10µL PIC. After final wash solution was removed, 35µL of 2X sample buffer was added to each tube and incubated at 95°C for 5 minutes. Samples were cooled then analyzed by Western blot analysis.

WCL's were prepared in identical manner for immunoprecipitation (IP). Following preparation of WCL, 2µL anti-TMIGD1 antibody was added to each WCL group and incubated for two hours at 4°C. Samples were then moved from refrigerator to ice tray and 50µL of protein-G agarose beads (purchased from EMD Millipore; cat#16-266) was added to each group. Samples were then incubated for an additional one hour at 4°C. After incubation, samples were centrifuged and supernatant was discarded prior to washing beads twice with EB buffer+10µL PIC and 1M NaCl. Solution was discarded after final wash, 2X sample buffer was added to beads and tubes were incubated at 95°C for 5 minutes. Samples were cooled then analyzed via Western blot analysis.

Fluorescent Microscopy

Two groups of cells were analyzed using a confocal microscope and fluorescent bulb. HEK-293T cells expressing TMIGD1 and EV were both transfected with GFP-tagged moesin using aforementioned technique. 72 hours after initial transfection, cells were washed with 5mL of PBS. PBS was aspirated and 1mL of trypsin was added for 45 seconds. Cells were washed off of plates using 8mL of DMEM with FBS and P/S. 2mL of cell-containing DMEM was transferred to a sterile 60mm tissue culture plate containing a cover slip. Cells were allowed to grow 48 hours until adhered to cover slip before images were captured using confocal microscope.

RESULTS

The discovery of TMIGD1 and its establishment as a novel adhesion molecule served as a starting point for elucidation of its undiscovered cellular mechanism. As a member of a class of molecules with numerous cellular functions, it came as little surprise that TMIGD1 itself served diverse roles in the cell, including modulating TEER, cell permeability, growth, adhesion, and actin arrangement.

Due to the common nature of Ig domains serving as mediators of protein interactions, GST pull-down assay was completed using extracellular TMIGD1 domains. Results of the assay showed formation of TMIGD1 complexes facilitated by Ig domain homodimerization. Further investigation revealed this homodimerization also facilitated cell adhesion of cells expressing TMIGD1 (Arafa et al., 2015).

Liquid Chromatography – Tandem Mass Spectrometry

With self-dimerization of extracellular Ig domains identified as putative initiator of function, focus shifted to the cytoplasmic domain of the protein. Using GST pull-down with cyt-TMIGD1, molecules binding to the cytoplasmic domain were isolated then analyzed through LC-MS/MS (unpublished data; courtesy Nader Rahimi, Kevin Chandler, 2015). LC-MS/MS data revealed several interactions, most notably moesin. Investigation of published data on moesin revealed its role in the adhesion, migration and actin arrangement in cells (Li et al., 2015). Due to the similarities between moesin and TMIGD1 in known cellular effect, moesin seemed to be a potential mediator of TMIGD1 function and was deemed worthy of further investigation.

GST Pull-down Assay

To investigate a potential relationship between TMIGD1 and moesin, GST pull-down assay was completed using specific domains of TMIGD1 and moesin. Initially, RKO cells expressing TMIGD1 and EV were lysed and incubated with GST control, GST-moesin (1-322) and GST-moesin (307-577). Moesin was separated into two fusion proteins to isolate the FERM domain (1-322) and the linker region plus C-ERMAD domain (307-577). This isolation pattern was selected to determine if the FERM domain alone was in fact the portion of moesin that binds to TMIGD1. Previous studies investigating the function of moesin indicated the N-terminus FERM domain was responsible for interacting with membranous protein in the cell while the C-ERMAD domain was responsible for interaction with cytoskeletal actin (Pearson, Reczek, Bretscher, & Karplus, 2000). Results of the GST pull-down assay supported this concept.

Western blot analysis in which C-MYC antibody was used to detect TMIGD1 following GST pull-down revealed a strong protein band at 45kDa in the group where WCL from RKO cells expressing TMIGD1 was incubated with fusion proteins containing the GST-moesin(1-322). Because 45kDa is the expected size of N-glycosylated TMIGD1 (Arafa et al., 2015), presence of this band indicated TMIGD1 binds the FERM domain of moesin, thereby preserving TMIGD1 on the fusion protein beads throughout the experiment and eluting the complex.

This data was corroborated by the presence of a strong band at 45kDa in the positive control group of WCL from RKO cells expressing TMIGD1. Lack of binding in the GST control group, as well as what was interpreted as only weak, nonspecific binding in the GST-moesin (307-577) group, further supported the specificity of moesin FERM domain binding to TMIGD1 (Figure 4).

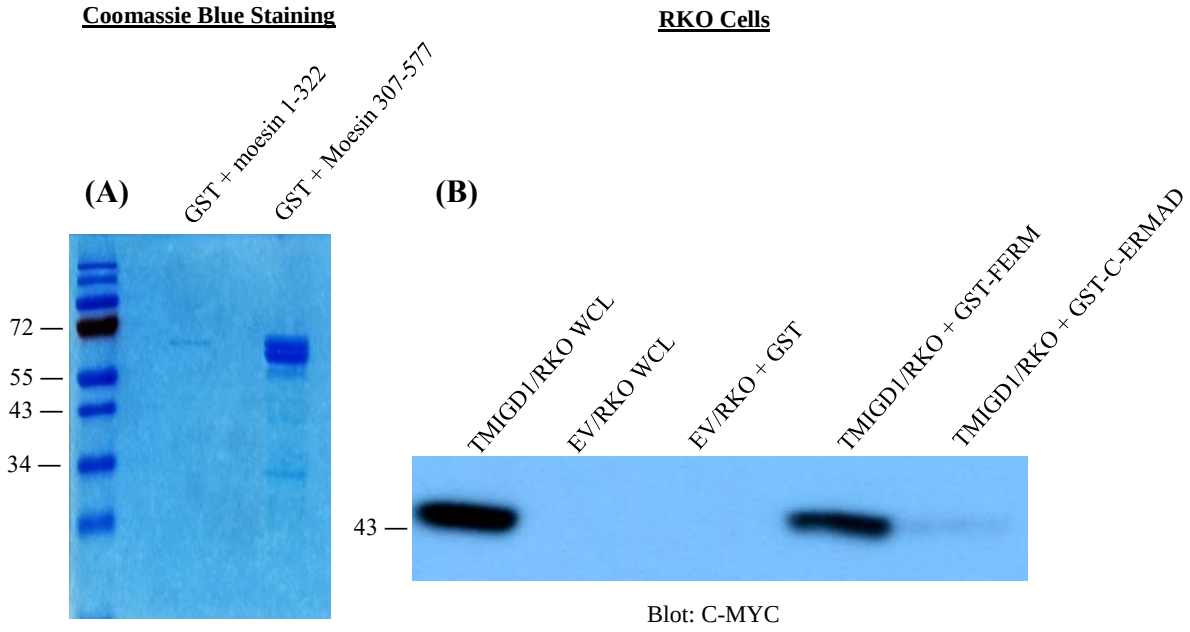

Figure 4. TMIGD1 Interacts With Moesin Via FERM Domain. (A) SDS-polyacrylamide gel stained with Coomassie Blue after electrophoresis of GST fusion proteins i) GST-moesin(1-322) ii) GST-moesin(307-577). (B) Whole cells lysates from rectal carcinoma cells (RKO) transduced to overexpress TMIGD1 (TMIGD1/RKO) and empty vector (EV/RKO) were incubated with GST-moesin fusion proteins. Fusion protein groups were i) moesin FERM domain (1-322) ii) moesin C-ERMAD domain (307-577) and iii) GST control (GST). Following WCL incubation, proteins were analyzed by Western blot analysis using C-MYC antibody to detect the C-MYC tag on TMIGD1.

Although the results of the GST pull-down assay supported the existence of a FERM domain-specific interaction between TMIGD1 and moesin, the single piece of data was insufficient to prove an interaction. The known tendency for GST to exhibit non-specific binding as well as the evidence that TMIGD1 may have numerous binding partners emphasized the need to obtain more evidence of this interaction. The next experimental method applied was to complete the GST pull-down assay again in essentially reverse order.

Initially, RKO cells expressing TMIGD1 and EV were prepared and lysed. Cyt-TMIGD1 fusion proteins were then added to WCL to pull-down moesin. The goal of this experiment was to elute moesin bound to cyt-TMIGD1 and detect using anti-moesin (38/87) antibody through Western blot analysis. Initial attempts to complete this assay were unsuccessful as no binding was detected in the sample groups and presence of moesin was not detected in the positive control group (unpublished data, 2016). The lack of moesin presence in the positive control group suggested moesin protein levels in RKO cells were too low to detect.

To resolve this issue, HEK-293T cells were transfected with GFP-tagged moesin and GFP-tagged EV control. GST pull-down assay with cyt-TMIGD1 was then attempted again with the transfected cells. Results of Western blot analysis using anti-moesin (38/87) antibody for detection revealed presence of a strong protein band at 77kDa in the group where cyt-TMIGD1 fusion protein beads were added to WCL, correlating to manufacturer specifications for moesin detection (Figure 5). Detection of moesin at this location supported the binding of cyt-TMIGD1 to moesin.

This was corroborated by the lack of binding in negative control groups and the presence of a strong protein band at 77kDa in the WCL positive control group. Slightly weaker bands were detected below the band representing moesin in both the experimental group and positive control group. These weaker bands were identical in location and relative size and were determined to be the result of minor moesin degradation during experimental procedures.

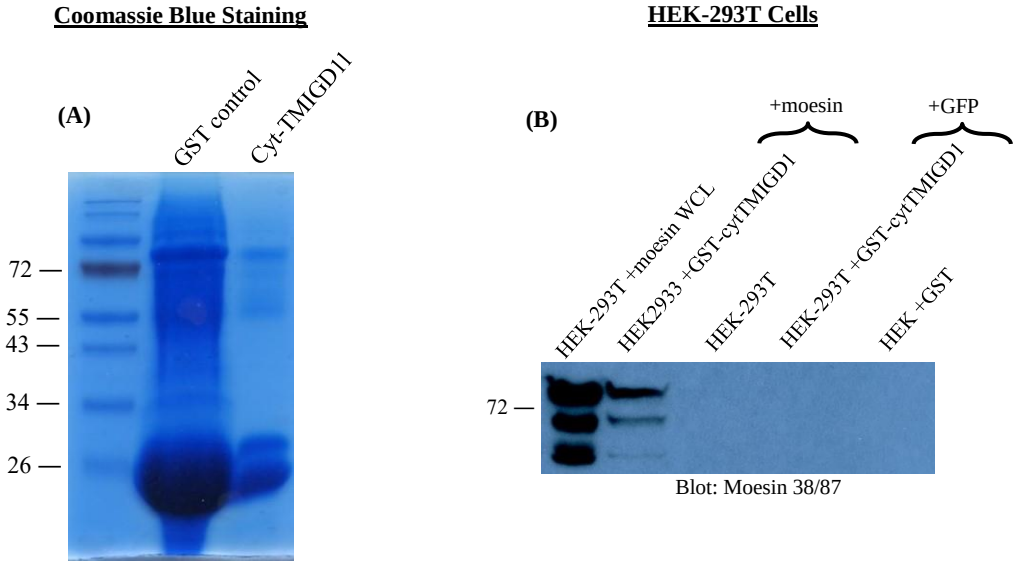

Figure 5. FERM Domain Interacts with Cytoplasmic TMIGD1. (A) SDS-polyacrylamide gel stained with Coomassie Blue after electrophoresis of cytoplasmic TMIGD1 bound to GST beads. (B) Whole cells lysates from human embryonic kidney cells (HEK-293T) expressing TMIGD1 (TMIGD1/HEK) and empty vector (EV/HEK) were transfected to with moesin (+moesin) or GFP control (+GFP). Following transfection, lysates were incubated with cytoplasmic TMIGD1-GST fusion proteins. Following WCL incubation, protein beads were analyzed by Western blot analysis using moesin (38/87) antibody.

Immunoprecipitation

The detection of binding through two different variations of GST pull-down assay, along with data obtained from LC-MS/MS, served as strong evidence supporting moesin FERM domain-specific binding to cyt-TMIGD1. Despite the convincing data already collected, it was determined that using another experimental procedure to demonstrate the relationship could ultimately be valuable. The final procedure employed to demonstrate binding was an IP of HEK-293T cells expressing TMIGD1 and EV that had also been transfected with GFP-tagged moesin and EV control.

Following preparation of WCL from each experimental group, anti-TMIGD1 (N8599) antibody and protein-G agarose beads were used to elute TMIGD1 bound to moesin. Beads were subsequently analyzed by Western blot analysis. Anti-C-MYC antibody was used to detect TMIGD1 and anti-GFP antibody was used to detect moesin.

Results showed presence of a strong protein band at 45kDa in the WCL positive control and both experimental groups expressing TMIGD1, indicating successful elution of TMIGD1 (Figure 6). This was corroborated by the absence of bands at 45kDa in the control groups not expressing TMIGD1. Strong protein bands were also detected at 105kDa in the WCL positive control and experimental group expressing TMIGD1 and overexpressing moesin. This band corresponded to the size of moesin bound to GFP and indicated moesin was also eluted in this experimental group since it was bound to TMIGD1. The existence of a much weaker band in the same location in the TMIGD1 group overexpressing GFP control was interpreted as the product of non-specific binding due to the high-affinity of cyt-TMIGD1 to binding.

Extremely weak, incomplete bands were detected at this location in the other control groups and were interpreted as non-specific binding. Due to the presence of this non-specific binding, the results of the IP experiment were deemed less convincing than the GST pull-down and LC-MS/MS data.

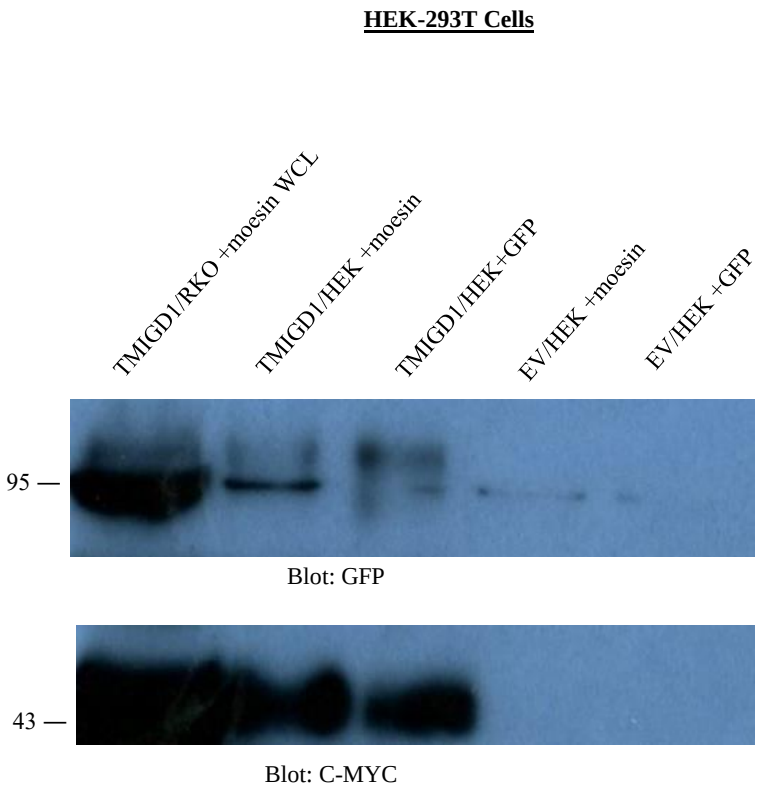

Figure 6. IP Demonstrates TMIGD1 Binding to Moesin FERM Domain. Whole cells lysates from human embryonic kidney cells (HEK-293T) expressing TMIGD1 (TMIGD1/HEK) and empty vector (EV/HEK) were transfected to with moesin (+moesin) or GFP control (+GFP). Following transfection, lysates were incubated with anti-TMIGD1 antibody and protein-G agarose beads. Protein-G beads were subsequently analyzed by Western blot analysis using GFP antibody and C-MYC antibody to detect GFP-tagged moesin and MYC-tagged TMIGD1, respectively.

Fluorescent Microscopy

With moesin FERM domain-specific binding of cyt-TMIGD1 established, investigation shifted to cellular effects of this interaction. The first step taken to determine functional relationship was to investigate cellular distribution of moesin with and without TMIGD1 expression. To accomplish this, HEK-293T cells expressing TMIGD1 and EV were transfected with GFP-tagged moesin. Following transfection, cells were transferred to a 60mm culture plate containing a microscope cover slip. Images taken with a confocal microscope were able to detect the GFP-tagged moesin under fluorescent light. The images revealed moesin distribution in HEK-293T cells was mostly peripheral but uniform in the absence of TMIGD1. In HEK-293T cells expressing TMIGD1, moesin was asymmetrically distributed in the cells. While moesin still exhibited a peripheral, juxtamembrane distribution, it appeared to be clustered in particular areas rather than uniformly distributed (Figure 7). Interpretation of this juxtamembrane distribution suggested moesin distribution was mediated by TMIGD1 and accumulations marked regions rich in TMIGD1.

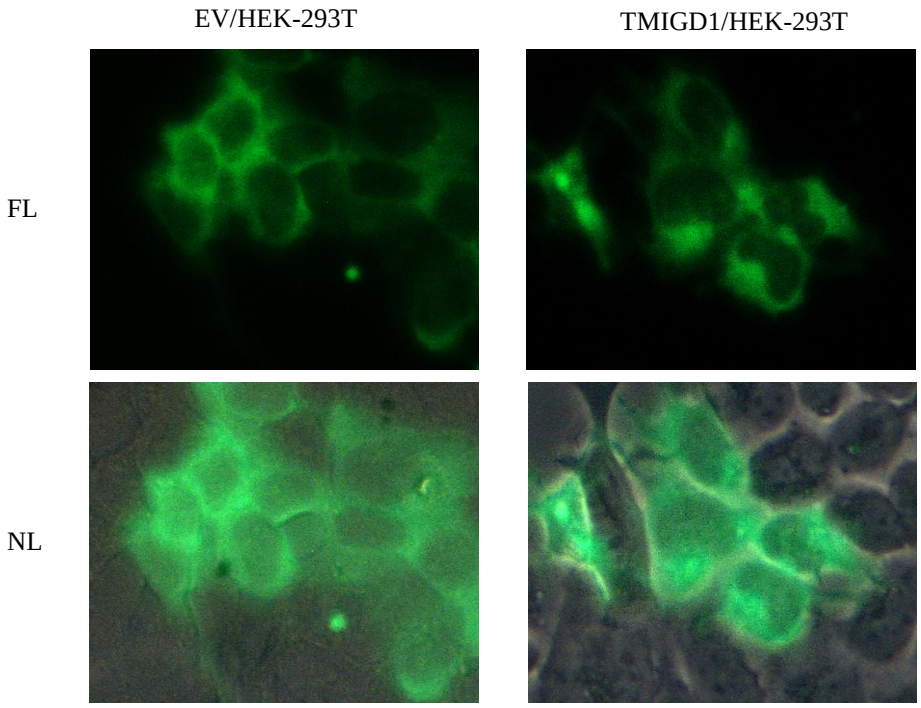

Figure 7. Moesin Distribution in Presence and Absence of TMIGD1. Images taken under normal light (NL) and fluorescent light (FL) to detect GFP-tagged moesin in HEK-293T cells transfected with moesin. Expression of TMIGD1 mediates moesin distribution as demonstrated by moesin clustering in particular areas, presumably TMIGD1-rich regions. EV cells display a uniform peripheral moesin distribution.

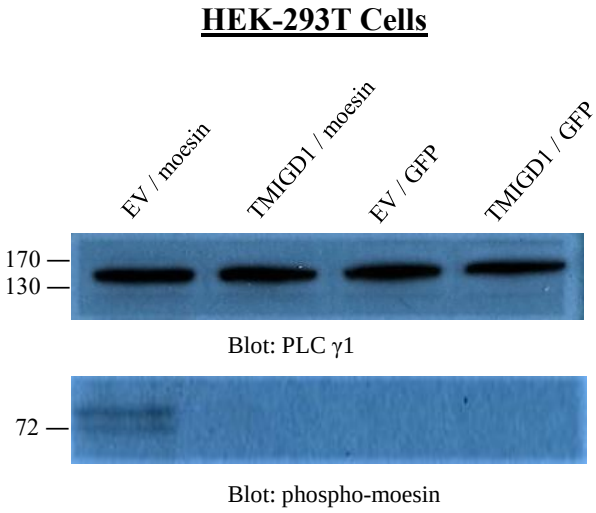

Figure 8: Detection of Phosphorylated Moesin in HEK-293T Cells. HEK-293T expressing TMIGD1 or empty vector (EV) that were transfected with moesin or GFP control (GFP) were analyzed by Western blot. Detection of phosphorylated moesin was accomplished using antibody specific for phosphorylated ERM proteins. Analysis revealed moesin is not phosphorylated when TMIGD1 is overexpressed while it is phosphorylated in the EV cells. Protein loading control was detected with anti-PLC γ1 antibody.

Phosphorylation Assay

After compiling information demonstrating TMIGD1 and moesin binding, as well as a functional relationship related to moesin distribution, focus moved to the functional mechanisms responsible. Activation of ERM family proteins has been studied in existing publications with focus on ERM protein phosphorylation. Phosphorylation of moesin in the cell is a critical step in the activation of the protein and its ability to mediate cellular changes (Q. Wang et al., 2016a). To investigate the phosphorylation status of moesin, a phosphorylation assay was completed using Western blot analysis. HEK-293T cells expressing TMIGD1 and EV were transfected with moesin and EV control and anti-phospho-ERM antibody was used to detect phosphorylated moesin. Results of the blot showed a strong protein band at 75kDa in the group containing TMIGD1 and moesin overexpression while all other groups showed no detectable bands (Figure 8). 75kDa is consistent with the size of moesin as indicated by manufacturer. Interpretation of this data suggested moesin is not phosphorylated when bound to TMIGD1, although it is phosphorylated in the absence of TMIGD1. Moesin not being phosphorylated in the presence of TMIGD1 means it is not active, establishing the possibility that TMIGD1 elicits its cellular effects by regulating moesin activity.

DISCUSSION

The discovery of TMIGD1 and subsequent investigation into its role as a CAM was enlightening, however, significant work is required to define its molecular mechanism of signaling in normal epithelial and tumor cells. TMIGD1 has a multi-faceted role in the cell, eliciting effects on adhesion, migration, TEER, permeability, growth rate and cytoskeletal actin arrangement, although modification of cytoskeletal actin may merely be how TMIGD1 elicits effects (Arafa et al., 2015). Elucidation of TMIGD1's extracellular domain mediating cell adhesion through homodimerization was a significant step in determining functional mechanism but offered little information regarding TMIGD1 intracellular signaling. LC-MS/MS analysis served as a crucial tool to start answering the question "how does TMIGD1 work inside the cell".

LC-MS/MS analysis of proteins eluted from WCL using the cytoplasmic domain of TMIGD1 provided a list of potential protein mediators for TMIGD1 function (Kevin Chandler et al., unpublished data). From this list, the protein moesin was of particular interest because of known roles and mechanisms of function in various cell systems.

Moesin has been shown to interact with transmembrane proteins, specifically adhesion molecules, and play a role in cell migration and actin arrangement (Hamada et al., 2000; Nam, Oh, Lee, Yoo, & Shin, 2015), making it a strong candidate to interact with TMIGD1 based on congruence of functional profiles.

Based on the congruence of apparent cellular functionality and supporting LC-MS/MS data, a hypothesis was formed suggesting moesin binds to cyt-TMIGD1 and mediates function (Figure 9).

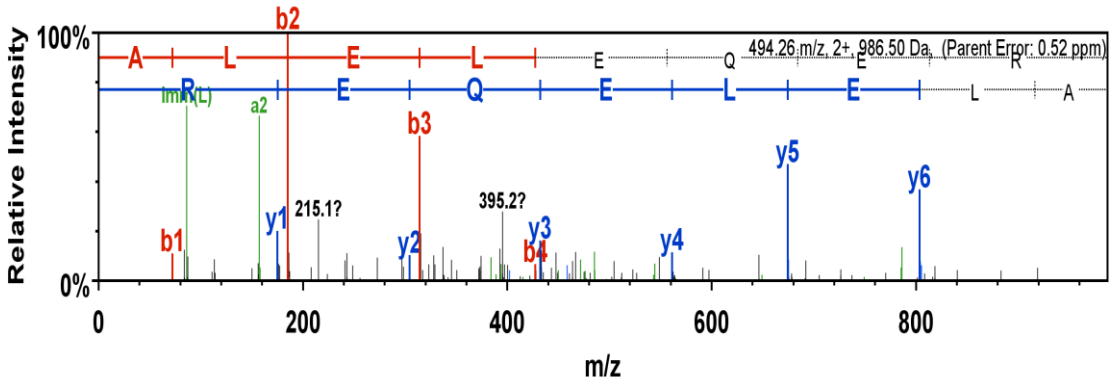

Figure 9: Results of LC-MS/MS Analysis. LC-MS/MS analysis revealed several possible binding partners to the cytoplasmic domain of TMIGD1. Among the possible partners was moesin. Further investigation of the TMIGD1 – moesin interaction was warranted due to similarities in function of the two proteins; specifically the relationship of both to F-actin (figure courtesy of Nader Rahimi, Kevin Chandler, 2015).

Establishment of Moesin FERM / TMIGD1 Binding

Experimental data obtained from GST pull-down assays supported the hypothesis by demonstrating binding between cytoplasmic TMIGD1 and the N-terminal FERM domain of moesin. The presence of this binding was initially indicated by GST pull-down using the FERM domain-specific portion of moesin to elute TMIGD1 from WCL and confirmed using the reverse method. The reverse method, a GST pull-down using cyt-TMIGD1 to elute moesin, yielded equally convincing results. Although the GST pull-down did in fact demonstrate binding and support the hypothesis, IP was used to alleviate any doubt and offer another means of demonstrating binding.

Despite the fact that IP results were less clear than the GST pull-down, data previously accumulated was sufficient to demonstrate strong, specific binding. Although this was the first time this binding had been demonstrated by anyone, data points were sufficiently convincing to warrant further investigation of TMIGD1 intracellular function and elucidation of a mechanism.

Moesin Mediates TMIGD1 Function

Establishment of moesin FERM domain binding to TMIGD1 provided insight into which protein TMIGD1 uses to elicit its effects but did not explain how binding elicits effect. A hypothesis regarding how binding elicits effect was formed based on data detailing independent effects of moesin and TMIGD1 in tissues. Several studies demonstrated moesin is upregulated in various cancers including breast, colon, cervical, skin and pancreatic (C. Y. Kim et al., 2012). Furthermore, moesin has been correlated with greater malignancy in tumors as its expression correlates with increased migration of cells, greater tumor invasiveness, greater MMP secretion, reduced cell-cell adhesion and reduced adherence to the ECM (Abiatari et al., 2010; Li et al., 2015). Of note is that moesin may play a role in propagating cell invasiveness in a feed-forward manner as MMP secretion leads to cleavage of CD44, subsequently freeing moesin into the cytoplasm where it may induce further invasive behavior (Kobayashi et al., 2004; Speck, Hughes, Noren, Kulikauskas, & Fehon, 2003a). Conversely, TMIGD1 is associated with greater cell adhesion, decreased migration and growth and is downregulated in human colon cancer cells (Arafa et al., 2015). This information taken together shaped the

hypothesis that TMIGD1 sequesters moesin in cancer cells, rendering moesin unable to elicit effects while simultaneously eliciting its own established effects.

To investigate this hypothesis, confocal microscopy was used to capture images of cellular moesin under fluorescent light. These images were used to determine differing moesin distribution in cells expressing TMIGD1 vs EV. Moesin in cells expressing TMIGD1 was localized to specific areas of the cell near the cell membrane, presumably areas rich in TMIGD1. EV cells showed a uniform, peripheral moesin distribution without any evidence of clustering. The contrast in moesin distribution patterns supported the hypothesis that TMIGD1 elicits effects by sequestering moesin and preventing it from being activated (Figure 10).

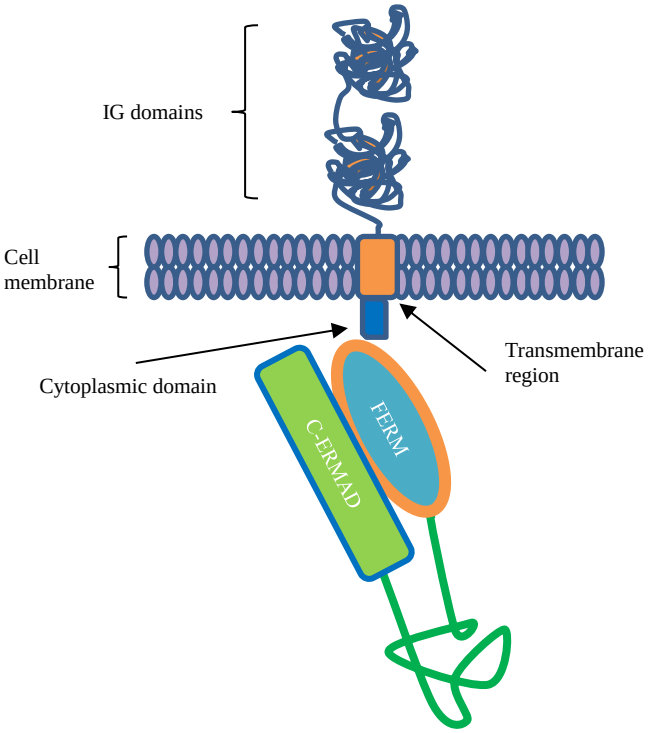

Figure 10: TMIGD Inhibits Moesin Phosphorylation. TMIGD1 binds the FERM domain of moesin. This interaction prevents phosphorylation and thereby activation of moesin in the cell. Moesin is phosphorylated in the absence of TMIGD1.

TMIGD1 Regulates Moesin Phosphorylation

Moesin localization imaging was useful for determining presence of an interaction but did not reveal mechanism of action. To determine mechanism responsible for TMIGD1 function as it relates to moesin binding, the phosphorylation of moesin was studied.

Phosphorylation of the moesin protein serves as the final activator of moesin and occurs via several pathways, including p38MAPK, Rho/ROCK and PKC pathways (Zhang et al., 2014). This phosphorylation however is seemingly not the most relevant part of the process in moesin activation as it pertains to TMIGD1. In its inactivated state, moesin is bound to itself and confined to the cytoplasm. In this state, moesin is in a conformation blocking the Thr558 residue that must be phosphorylated to confer activity. Systematic binding of PIP2 induces a series of events that eventually lead to conformational change exposing Thr558 for phosphorylation (Ben-Aissa et al., 2012). It was hypothesized that TMIGD1 sequestering prevents moesin from getting all the way to the cell membrane where it can undergo conformational changes induced by PIP2.

Phosphorylation assay was conducted using anti-phospho-ERM antibody to detect phosphorylation states of moesin in experimental groups. Results supported the hypothesis by showing moesin was phosphorylated in in the absence of TMIGD1 expression but not when cells expressed TMIGD1. This piece of data confirms TMIGD1 prevents the phosphorylation of moesin, thereby suppressing the effects of moesin and exerting the observed effects of TMIGD1. This data is consistent with the hypothesis that

TMIGD1 sequesters moesin, preventing interaction with PIP2. Without PIP2 interaction, moesin cannot interact with CAM's such as ICAM's, CD44, integrins or E-cadherins.

In conclusion, data presented in this work demonstrates a strong and specific binding between TMIGD1 and the FERM domain of moesin. This binding, which was demonstrated by three different methods, appears to prevent moesin activation. TMIGD1 sequesters moesin to specific cellular regions, likely to adherens junctions, where TMIGD1 is localized, preventing its interaction with PIP2. In this manner, TMIGD1 alters the balance of moesin activation in the cell and limits effects of moesin, explaining the protective properties of TMIGD1 demonstrated experimentally by Arafa et al. (2015). Furthermore, this dynamic explains the benefit of downregulation of TMIGD1 to tumor cells. Downregulation of TMIGD1 in aberrantly growing cells essentially removes moesin's counterbalance, leaving the deleterious effects of moesin unchecked. With this in mind, experimental design allowing control of PIP2 in this process should be employed to further investigate the mechanism of TMIGD1 in the context of tumor cells.

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